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BIOLOGIC RESPONSE MODIFIERS

Effect of Antimicrobial Agents on Inflammatory Cytokines in Acute Leptospirosis

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ABSTRACT The aim of this study was to assess the inflammatory cytokine response and possible association with antimicrobial treatment with penicillin, ceftriaxone, and doxycycline in acute leptospirosis. In the early acute stage, interleukin-10 (IL-10) levels were higher in mild cases than in severe cases ($P = 0.01$). IL-6 and IL-8 levels were low in patients who received >5 antimicrobial doses ($P < 0.01$). IL-8 levels were negatively correlated with the number of ceftriaxone doses administered ($r = -0.315$; $P = 0.031$). Further studies are needed to evaluate the possible downregulation of proinflammatory cytokines by ceftriaxone in leptospirosis.

KEYWORDS ceftriaxone, inflammatory cytokines, interleukin-8, leptospirosis

Leptospirosis has the highest annual global incidence among zoonotic infections. Previous studies have shown altered tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), IL-6, IL-8, IL-10, and IL-12 levels in leptospirosis (1, 2). The severity of disease in leptospirosis is associated with a cytokine storm, with high levels of TNF-α, IL-6, and IL-10 (2).

Penicillin (PEN), ceftriaxone (CRO), doxycycline (DOX), and cefotaxime are common antimicrobial agents used for the treatment of leptospirosis. Early treatment is effective in resolving clinical manifestations and promoting recovery. However, studies on the possible effects of antimicrobials on inflammatory cytokine responses in leptospirosis are limited. The main aims of this study were to assess the inflammatory cytokine levels and to determine the effects of PEN, DOX, and CRO on plasma cytokine levels in patients with acute leptospirosis.

This study received ethics approval from the Ethics Review Committee, Faculty of Medicine, University of Colombo (protocol no. EC-12-056). The study protocol did not include withholding antimicrobial treatment prior to recruitment to the study. During the period from April 2013 to November 2014, a total of 545 patients with clinically suspected leptospirosis were recruited from two hospitals, the National Hospital and Base Hospital Homagama, in Sri Lanka. Laboratory confirmation of leptospirosis was performed using the microscopic agglutination test (3). Other inclusion criteria were defined based on the stage of antimicrobial treatment at the time of recruitment to the study, i.e., no treatment received or treatment received for not more than 48 h. Accordingly, 51 patients with confirmed leptospirosis were selected; all were survivors. Plasma was separated, aprotinin (0.5 TIU) was added, and the samples were stored at −80°C. Disease severity was determined as severe or mild as described previously (3) (see Table S1 in the supplemental material). Plasma samples obtained from age- and

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gender-matched healthy individuals were used as controls. Written informed consent was obtained from all patients and healthy control subjects prior to recruitment into the study.

Plasma TNF-α, IL-1β, IL-6, IL-8, IL-10, and IL-12p70 levels were measured using an inflammatory colorimetric bead array (BD Biosciences, San Jose, CA), following the manufacturer's instructions, with a FACSCalibur flow cytometer (BD Biosciences). CellQuest software and FCAP software were used to analyze the data, to generate the results in different modes (2). The minimal level of detection was 0.1 pg/ml for each cytokine.

Patients ($n = 51$) had significantly higher plasma levels of IL-6 (39.40 $\pm$ 83.07 pg/ml), IL-8 (25.83 $\pm$ 35.16 pg/ml), and IL-10 (21.67 $\pm$ 68.69 pg/ml) than did healthy controls ($n = 18$) (0.06 $\pm$ 0.19, 0.13 $\pm$ 0.35, and 0.00 pg/ml, respectively; $P < 0.001$). The elevated cytokine levels in leptospirosis patients, compared to healthy controls, reported here were similar to those observed previously in Indonesia (4). These cytokine levels were also similar to those of the survivors of severe leptospirosis (SL) described by Reis et al. (2). The present study showed a positive correlation between plasma IL-6 levels and plasma IL-8 levels ($n = 51$, $r = 0.734$; $P < 0.001$). TNF-α, IL-1β, and IL-12p70 levels were undetectable in all patients and healthy controls except for two patients with SL (IL-12p70 levels of 8.2 and 18.5 pg/ml). In addition to IL-10, the potential of IL-6 to inhibit TNF-α and IL-1 was reported previously (1). Therefore, it is possible that the absence of detectable levels of TNF-α and IL-1 β may be due to the elevated levels of IL-6 and IL-10 detected. Conversely, it is unlikely that the undetectable levels of TNF-α, IL-1β, and IL-12p70 are due to storage at -80°C without freeze-thaw cycles (5).

Of the 51 patients, 26 were clinically categorized as having mild leptospirosis (ML) and 25 as having SL. Clinical characteristics of the patients are presented in Table S1 in the supplemental material. The cytokine profiles were similar for SL and ML patients. However, when the patients whose plasma samples were collected during the early acute phase of illness ($\leq$7 days) were considered (19 patients with ML and 15 with SL), a significant difference with respect to IL-10 levels was observed. ML patients had significantly higher IL-10 levels (47.98 $\pm$ 78.08 pg/ml; $P = 0.01$), compared to SL patients (0.93 $\pm$ 1.79 pg/ml). However, IL-6 and IL-8 levels were similar (IL-6, 57.91 $\pm$ 87.67 and 12.75 $\pm$ 13.47 pg/ml, respectively; IL-8, 36.17 $\pm$ 35.74 and 17.72 $\pm$ 16.23 pg/ml, respectively) (Table S1). Compared to previous studies, ML patients in this study had similar IL-10 levels and lower proinflammatory cytokine levels (1). The significantly higher anti-inflammatory cytokine (IL-10) levels and the trends observed with low IL-6/IL-10 ratios and low IL-8/IL-10 ratios could have played a role in preventing disease progression in ML patients. Significantly higher proinflammatory/anti-inflammatory cytokine ratios have been reported previously, i.e., in severe malaria and other diseases (6).

The effects of antimicrobial treatment on cytokine levels in leptospirosis have not been reported previously. Therefore, we analyzed the cytokine levels in patients who were in the early versus late treatment phase (0 to 5 doses [$n = 30$] versus $>$5 doses [$n = 21$]) at the time of sampling (Table S2). Interestingly, patients who had received $>$5 doses of antimicrobial drugs showed significantly lower IL-6 (13.01 $\pm$ 31.16 versus 54.24 $\pm$ 100.52 pg/ml; $P = 0.005$) and IL-8 (8.66 $\pm$ 12.51 versus 34.37 $\pm$ 40.01 pg/ml; $P = 0.001$) levels, compared to patients who had received 0 to 5 doses. However, IL-10 levels were similar for the two groups (1.56 $\pm$ 3.90 versus 23.16 $\pm$ 65.40 pg/ml; $P = 0.107$), indicating that the number of antimicrobial doses had a minimal effect on plasma IL-10 levels. Of the three cytokines, IL-8 levels were negatively associated with the total number of antimicrobial doses administered (cumulative effects of CRO, PEN, and DOX) ($r = -0.327$; $P = 0.025$) and also with the number of CRO doses administered ($r = -0.315$; $P = 0.031$) (Fig. 1A and B). However, such a negative correlation was not observed with either PEN or DOX alone (Fig. 1C and D). Moreover, other CRO combinations, i.e., with either PEN or DOX, did not show such a negative correlation with IL-8 levels, demonstrating that none of the combined doses was significantly associated with IL-8 levels. Furthermore, this finding may indicate that the negative effect on IL-8 levels may be specific for CRO.

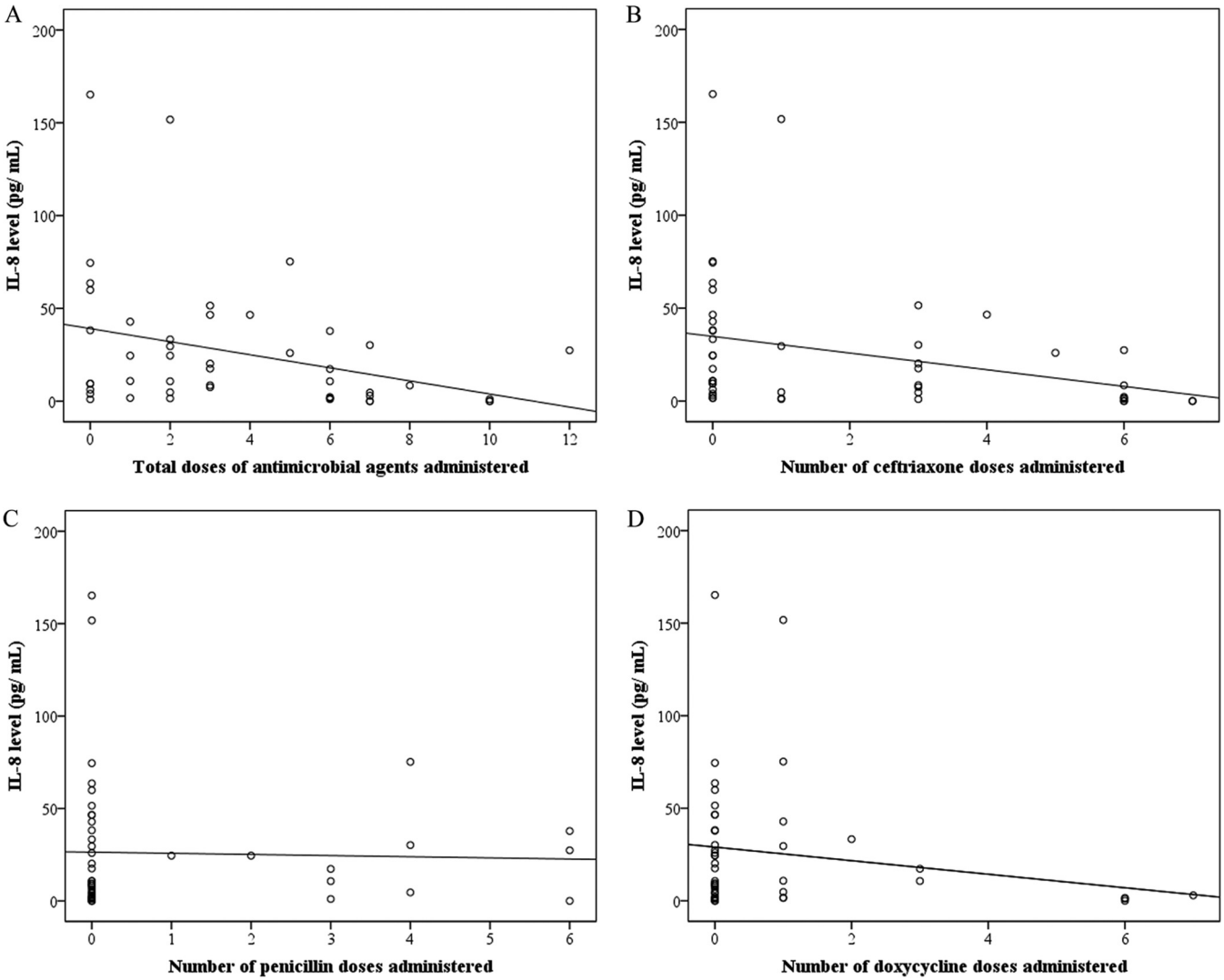

FIG 1 Associations of IL-8 levels with the numbers of antimicrobial doses administered. The levels of IL-8 in relation to the total number of antimicrobial doses administered (A), the number of CRO doses administered (B), the number of PEN doses administered (C), and the number of DOX doses administered (D) are shown. The fit lines represent the associations of IL-8 levels with the antimicrobial doses administered. Each dot in the scatter plots represents the IL-8 level at the corresponding antimicrobial dose number.

The negative correlation observed between IL-8 levels and antimicrobial treatment, more specifically with the number of CRO doses administered, may be due to downregulation of the inflammatory cytokine response. It is noteworthy that *in vitro* downregulation by CRO of the production of proinflammatory cytokines, including IL-8, induced by *Leptospira* in peripheral blood mononuclear cells has not been investigated. CRO was not shown to downregulate the production of IL-8 in peripheral blood mononuclear cells in response to lipopolysaccharide (LPS) and lipoteichoic acid, *Staphylococcus pneumoniae*, and *Haemophilus influenzae*, although the same study showed inhibitory activity for trovafloxacin (7). There are supportive *in vitro* data to show that DOX can also downregulate the production of TNF-α, IL-1, and IL-6 induced by staphylococcal exotoxins in peripheral blood mononuclear cells (8). Further, this effect on IL-8 has not been investigated. The results of the present study, however, do not reflect downregulatory effects of DOX on any of the proinflammatory cytokines tested.

The observed lack of negative correlation between IL-8 levels and PEN may be due to the association between PEN and the Jarisch-Herxheimer reaction reported for leptospirosis (9). No such risk has been reported for CRO, which may corroborate the observed negative correlation between IL-8 levels and CRO. Our findings allow us to

hypothesis that CRO may be effective in decreasing IL-8 levels. Therefore, we emphasize the need for further studies to evaluate the effects of CRO in downregulating inflammatory cytokine responses in leptospirosis.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02312-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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N.F., R.D.S., S.M.H., H.J.D.S., S.R., and S.P. conceived and designed the experiments, N.F., R.D.S., and L.K. performed the experiments, N.F., S.M.H., and S.R. analyzed the data, N.F., S.M.H., S.R., and S.P. wrote the paper, and N.F., R.D.S., S.M.H., L.K., N.L.D.S., H.J.D.S., S.R., and S.P. critically reviewed and revised the draft and approved the final manuscript.

REFERENCES

1. Papa A, Kotrotsiou T. 2015. Cytokines in human leptospirosis. Trans R Soc Trop Med Hyg 109:749–754. https://doi.org/10.1093/trstmh/trv095.
2. Reis EA, Hagan JE, Ribeiro GS, Teixeira-Carvalho A, Martins-Filho OA, Montgomery RR, Shaw AC, Ko AI, Reis MG. 2013. Cytokine response signatures in disease progression and development of severe clinical outcomes for leptospirosis. PLoS Negl Trop Dis 7:e2457. https://doi.org/10.1371/journal.pntd.0002457.
3. Fernando N, Wickremesinghe S, Niloofa R, Rodrigo C, Karunanayake L, de Silva HJ, Wickremesinghe AR, Premawansa S, Rajapakse S, Handunnetti SM. 2016. Protein carbonyl as a biomarker of oxidative stress in severe leptospirosis, and its usefulness in differentiating leptospirosis from dengue infections. PLoS One 11:e0156085. https://doi.org/10.1371/journal.pone.0156085.
4. Wagenaar JF, Gasem MH, Goris MG, Leeflang M, Hartskeerl RA, van der Poll T, van 't Veer C, van Gorp ECM. 2009. Soluble ST2 levels are associated with bleeding in patients with severe leptospirosis. PLoS Negl Trop Dis 3:e453. https://doi.org/10.1371/journal.pntd.0000453.
5. de Jager W, Bourcier K, Rijkers GT, Prakken BJ, Seyfert-Margolis V. 2009. Prerequisites for cytokine measurements in clinical trials with multiplex immunoassays. BMC Immunol 10:52. https://doi.org/10.1186/1471-2172-10-52.
6. Perera MK, Herath NP, Pathirana SL, Phone-Kyaw M, Alles HK, Mendis KN, Premawansa S, Handunnetti SM. 2013. Association of high plasma TNF-alpha levels and TNF-alpha/IL-10 ratios with TNF2 allele in severe *P. falciparum* malaria patients in Sri Lanka. Pathog Glob Health 107:21–29. https://doi.org/10.1179/2047773212Y.0000000069.
7. Purswani M, Eckert S, Arora H, Johann-Liang R, Noel GJ. 2000. The effect of three broad-spectrum antimicrobials on mononuclear cell responses to encapsulated bacteria: evidence for down-regulation of cytokine mRNA transcription by trovafloxacin. J Antimicrob Chemother 46:921–929. https://doi.org/10.1093/jac/46.6.921.
8. Krakauer T, Buckley M. 2003. Doxycycline is anti-inflammatory and inhibits staphylococcal exotoxin-induced cytokines and chemokines. Antimicrob Agents Chemother 47:3630–3633. https://doi.org/10.1128/AAC.47.11.3630-3633.2003.
9. Raptis L, Pappas G, Akritidis N. 2006. Use of ceftriaxone in patients with severe leptospirosis. Int J Antimicrob Agents 28:259–261. https://doi.org/10.1016/j.ijantimicag.2006.04.011.